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Identification of minimum Rpn4-responsive elements in genes related to proteasome functions☆

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ABSTRACT

The proteasome is an essential, 66-subunit protease that mediates ubiquitin-dependent proteolysis. The transcription factor Rpn4 regulates concerted expression of proteasome subunits to increase the proteasome by recognizing nonamer proteasome-associated control element (PACE) elements on the promoter regions. However, the genes for proteasome assembly chaperones and some of the subunits have no PACEs. Here we identified a minimal hexamer "PACE-core" sequence that responds to Rpn4. PACE-cores are found in many genes related to proteasome function including the assembly chaperones, but cannot substitute for PACE of the subunits. Our results add a new layer of complexity in transcriptional regulation of genes involved in protein degradation.

1. Introduction

The 26S proteasome is a huge protease complex conserved among eukaryotes. It degrades proteins tagged with ubiquitin chains that serve as recognition motifs for targeting to the proteasome. This selective protein degradation plays essential roles in regulating various cellular activities such as cell cycle progression, stress response, transcription, and signal transduction [1,2]. Consequently, maintaining proper proteasome activity is vital for cellular proliferation and homeostasis.

The 26S proteasome consists of 33 different subunits and can be divided into two complexes; a 20S core particle (CP) and a 19S regulatory particle (RP). The CP exerts proteolytic activity and is made up of axial stacking of four hetero-heptameric rings; two outer α-rings formed by α1–α7 and two inner β-rings formed by β1–β7. The RP contains six AAA-ATPases Rpt1–6 and 13 non-ATPase subunits Rpn1–3, Rpn5–13, and Sem1/Rpn15 [3]. In addition, at least nine conserved proteasome assembly chaperones are required for efficient formation of the proteasome [4–9]; Pba1–4/PAC1–4 and Ump1/POMP mediate CP assembly, and Nas2/p27, Nas6/p28, Hsm3/S5b, and Rpn14/PAAF are dedicated to the RP assembly in yeast/mammals.

In *Saccharomyces cerevisiae*, Rpn4 is a transcription factor that activates the concerted expression of proteasome subunits and thus promotes *de novo* formation of the proteasome [10,11]. Rpn4 is a short-lived protein degraded by the proteasome in both ubiquitin-dependent and -independent manners [12]. When proteasome activity is compromised, Rpn4 is stabilized and accumulates in cells, leading to potent induction of gene expression of the proteasome subunits. Thus, the abundance and activity of the proteasome are regulated in an Rpn4-dependent feedback circuit [13].

Previous studies have shown that Rpn4 binds to a nonamer box 5′-GGTGGCAAA-3′ called *p*roteasome-*a*ssociated *c*ontrol *e*lement (PACE), which is found in the promoter regions of most proteasome subunit genes [10,14]. However, some of the proteasome subunit genes such as *α6/PRE5*, *β2/PUP1*, *RPN8*, *RPN10*, *RPN13*, and *SEM1* do not have this authentic PACE sequence in their promoter regions. Instead, octamer PACE-like sequences, 5′-AGTGGCAA-3′, or 5′-GGTGGCGA-3′ were found in promoter regions of these genes, although whether it responds to Rpn4 was not determined [15,16]. In addition, there are no reports regarding PACE or PACE-like sequences in the promoters of genes for the assembly chaperones. Because the chaperones are required for efficient proteasome

Abbreviations: PACE, proteasome-associated control element; CP, core particle; RP, regulatory particle; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin; $PACE^{var}$, variant PACE; $PACE^{core}$, PACE-core; GET, guided entry of tail-anchored proteins; ChIP, chromatin immunoprecipitation; AZC, azetidine-2-carboxylic acid

☆ *Author contributions:* R.S. and S.M. designed the research and analyzed the data. R.S. and H.Y. performed the experiments. R.S. and S.M. wrote the manuscript.

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assembly, it would be reasonable to assume that they are under the same regulatory mechanism. At present, it is not known whether Rpn4 induces transcription of the assembly chaperone genes, which do not have the authentic PACE sequence.

In this study, we examined the Rpn4-dependent transcription of proteasome subunit and chaperone genes and found that Rpn4 induces the transcription of genes that do not have the reported consensus sequences. Through these analyses, we identified a minimum six-base sequence that responds to Rpn4.

2. Materials and methods

2.1. Yeast strains and media

BY4741 and BY4743 (Open biosystems) were used as wild-type strains. Heterozygous diploids of *α4* and *RPN6* were obtained from Yeast Heterozygous Collection (Openbiosystems). $\alpha4_{int}$-cells/YWD438 and $\alpha4^{*}_{int}$-cells/YWD439 were established by using the plasmids pUZ198 and pUZ199 as described below. Media and methods for standard culture, sporulation, tetrad analysis, and transformation were performed as described previously [17].

2.2. Plasmid construction

To overexpress *RPN4*, *RPN14*, and *NAS6*, the open reading frames (ORFs) of *RPN4*, *RPN14*, and *NAS6* were amplified from the yeast genome and cloned into pYF5, which is a vector modified from pYES2 (Invitrogen) for galactose-driven overexpression [18].

For chromatin immunoprecipitation (ChIP) assay, we created a galactose-driven 3xFlag vector pUZ251 by modifying pYF5. The *RPN4* ORF was cloned from pMO49 into pUZ251, resulting in pUZ252.

For conversion of PACE sequences of *α4*/*PRE6* and *RPN6* into the PACE-core sequence of *PBA2*, plasmids were constructed as follows: *α4* and *RPN6* genes were amplified from the yeast genome. The amplified fragments were then cloned into a pBluescript SK- vector. PACE sequences were mutagenized, and the fragments were cloned into pRS315 [19]. Resultant plasmids were used for transcriptional analysis. To replace the endogenous PACE sequence of *α4* with the PACE-core motif of *PBA2*, *Xho*I-*Bgl*II fragments of pUZ195 and pUZ192 were cloned into the *Xho*I-*Bam*HI sites of pRS305 [19]. Resultant plasmids, pUZ198 and pUZ199, were linearized by *Bam*HI digestion and introduced into BY4743 cells. Transformed cells were sporulated and segregated. Plasmids used in this study are shown in Table 1.

Table 1
Plasmids used in this study.

Plasmid name	Vector	Insert
pMO49	pYF5	*RPN4*
pUZ177	pYF5	*NAS6*
pUZ178	pYF5	*RPN14*
pUZ183	pBluescript	*α4*
pUZ184	pBluescript	*RPN6*
pUZ186	pBluescript	*α4**
pUZ187	pBluescript	*RPN6*
pUZ192	pRS315	*α4**
pUZ193	pRS315	*RPN6*
pUZ194	pRS315	*RPN6**
pUZ195	pRS315	*α4*
pUZ198	pRS305	*α4*
pUZ199	pRS305	*α4**
pUZ251	pYF5	(3xFlag vector)
pUZ252	pUZ251	*RPN4*
pUZ293	pYC2/CT/*lacZ*	3xPACE-*CYC1 promoter*
pUZ294	pYC2/CT/*lacZ*	3xPACE^{var}-*CYC1 promoter*
pUZ295	pYC2/CT/*lacZ*	3xPACE^{core}-*CYC1 promoter*
pUZ296	pESC-LEU	*RPN4*

2.3. Biochemical analysis

Yeast cells harboring galactose-inducible plasmids were incubated in SGal medium lacking uracil for 2 h. Protein extraction, glycerol gradient centrifugation, and assays for peptidase activity were performed as described previously [20].

2.4. Western blotting

Western blotting was performed using anti-Pgk1 antibody (Invitrogen, 429250) and polyclonal anti-β5/Pre2 and anti-Rpn2 rabbit antisera as primary antibodies, and HRP-conjugated anti-mouse IgG and anti-rabbit IgG as secondary antibodies (Jackson ImmunoResearch).

2.5. Quantitative RT-PCR

Yeast cells harboring galactose-inducible plasmids were incubated in SGal medium lacking uracil for 30 min. Yeast cells were collected and disrupted with glass beads, and total RNA was extracted using a High Pure RNA isolation kit (Roche). The RNA was reverse-transcribed using a SuperScript VILO cDNA synthesis kit (Invitrogen), and the relative amounts of cDNA for each target gene were quantified using a Light Cycler 480 and Universal ProbeLibrary (Roche). The primers and probe numbers are listed in Table 2. *ACT1* was used as a normalization control.

2.6. Chromatin immunoprecipitation (ChIP)

Yeast cells harboring galactose-inducible plasmids were incubated in SGal medium lacking uracil for 30 min. ChIP assay was performed as described previously [21]. The amount of precipitated DNA was quantified using the Light Cycler 480 system (Roche). The primers and probe numbers are described in Table 2.

3. Results and discussion

3.1. Overexpression of Rpn4 increases proteasomes

Previous reports have described that Rpn4 is required for maintaining normal proteasome activity, especially when the proteasome activity is impaired, and thus indispensable for the viability of proteasome mutants in yeast [11,22]. To see whether ectopic overexpression of Rpn4 increases functional proteasomes even in wild-type cells, which already have enough proteasomes, we examined the activity and amount of proteasomes in cells overexpressing Rpn4. Rpn4 expression increased the amount of the CP subunit β5/Pre2 and the RP subunit Rpn2 (Fig. 1A). To confirm that the increased proteasome subunits were properly assembled, cell lysates were separated by glycerol gradient centrifugation, followed by measurement of proteasome activity and Western blot. The proteasome activity was increased in both the 20S CP fractions (fractions 16–20) and the 26S proteasome fractions (fractions 24–30) by Rpn4 overexpression, compared to control cells (Fig. 1B). In accordance with this activity, the protein amount of β5 was increased in both the 20S and 26S fractions (Fig. 1B). Rpn2 was also increased in the 26S fractions (Fig. 1B). These results are consistent with a previous study showing that Rpn4 overexpression increased levels of proteasome proteins [22] and suggest that Rpn4 overexpression induces the expression of all genes required for the construction of properly assembled functional proteasomes, even in wild-type cells with normal proteasome activity.

Table 2
Primers and Probes used for quantitative PCR.

Gene name	Forward	Reverse	Probe
ACT1	ccatcttccatgaaggtcaag	ccaccaatccagacggagta	25
α6/PRE5	tggctccagatgctagagttt	cggcaagctttctgttaaaaa	78
α7/PRE10	ggtataaagtgtaacgacggtgtagtc	tgtgggaccagcagtttaga	86
RPN8	ggtgtagaacacttattgagagacgta	ttggttggtcaaccggatag	62
RPN10	gacttcggggtagacccatc	cttccatagacagacgcaagg	115
RPN12	ccagaaagcatgggatcttc	ttaggatatcggtaaaagagtcgaa	115
UMP1	ccacacaattgaatgacagaca	atttgtctttggcgctgtgt	48
PBA1	cattgacggtgaaaagtcca	cgtcgtaattccacgagtgtt	60
PBA2/ADD66	gggaatataccacaattgagtattga	ttcccattcatttgcttgtg	66
PBA4/POC4	gattagccgaactattgaatcagaa	tcgtctgcaggtagagtagcaa	42
NAS2	ctagaggcgtatttcagtgtgc	tcaccaacgcagagtccat	132
NAS6	tggtgcagaatatgatcttgtagata	ttaacctgttcattaagggcaac	29
RPN14	cgacaccagtattaagcttgacattat	tgggcgtctcaaattcaatag	115
HSM3	aaaatttctgctcaatgagatgc	gcgctcccatcacctatc	44
DDI1	aacagcttatggatctaggtttcc	tctgcatttccgttagtctgttt	89
UBP6	tgttgcagacatgcttaccc	tttcaacttttctcagttcgtca	84
GET3	agatttccatgtcgttaaaatgc	tttgtttaggaactgtgagaactttg	67
BNI1	tggttatttgccgttgaaca	cgcctactaaggaacccattt	144
PRB1	tcaaaggttccacagccaat	aacagccaagtccaaagctg	77
HSC82	ccaagtggagaaggttgttgt	ccgaattgaccagttctgatg	78
HSP82	tggcctacccaatccaatta	tcatccttcttttcctcgtctt	143
RPN12promoter	tgctagtgcccaatcaaaca	atttaagttacaggctagcgaaaaa	122
PBA2promoter	gagcgtggatcgttcactg	ccagtcaaatcgaggaatgc	146
HSM3promoter	gaaaagaaaaaggacgaagataaaaa	ccagctataacaaaaatccatgtaaa	135

3.2. Rpn4 promotes transcription of genes with variant PACE sequences

Previous studies reported PACE-like sequences in promoter regions of *α6/PRE5*, *β2/PUP1*, *RPN8*, *RPN10*, *RPN13,* and *SEM1*, which do not have the authentic PACE sequences [15,16,23]. We noticed that these subunit genes share a consensus nonamer sequence 5′-(A/G)GTGGC(A/G)AA-3′ that is different from the authentic PACE in the first and/or the seventh nucleotide (Table 3; italicized and underlined). We hereafter refer to these nonamer sequences as variant PACE (PACE^{var}) sequences to discriminate from the authentic PACE sequence. Although both PACE^{var} and the authentic PACE sequences fit the Rpn4-recognision motif in the JASPAR database, 5′-(A/G/T)G(T/G)GGC(A/G)-3′ [24,25], the PACE^{var} sequences have not been experimentally examined for whether they really mediate Rpn4-dependent gene expression, similar to the authentic PACE sequence.

To examine whether Rpn4 also regulates transcription of genes with the PACE^{var} sequences, we quantified the mRNAs for proteasome subunit genes that have the authentic nonamer PACE (*α7/PRE10* and *RPN12*) and genes that have the PACE^{var} sequences but do not have the authentic PACE (*α6/PRE5*, *RPN8*, and *RPN10*), with or without induction of Rpn4 expression. The mRNAs of PACE^{var}-containing genes were upregulated at levels comparable to those of the authentic PACE-containing genes (Fig. 1C). These results suggest that Rpn4 promotes transcription of all proteasome subunit genes with either the authentic PACE or the PACE^{var} sequence.

3.3. Rpn4 activates transcription of assembly chaperone genes that have neither PACE nor variant PACE sequences

As shown in Fig. 1A–C, Rpn4 overproduction increased mRNA levels of proteasome subunits and hence increased the amount of assembled proteasomes. Since the nine chaperones dedicated to proteasome assembly are needed for efficient proteasome biogenesis [3,26], it would be advantageous if these assembly chaperones were also upregulated by Rpn4 along with proteasome subunits. However, none of the assembly chaperones have either PACE or PACE^{var} sequences in their promoter regions, except for *NAS6*, which has a PACE^{var} sequence (Table 3). We examined whether the expression of the assembly chaperones responds to Rpn4 overexpression. We found that most of the chaperone genes were upregulated by Rpn4, although the extents varied (Fig. 1D). While the effect on *PBA4* expression was only modest, expression of *NAS2*, *HSM3*, *RPN14*, and *NAS6* was moderately increased, and expression of *PBA1*, *PBA2/ADD66*, and *UMP1* was strongly induced (Fig. 1D). These results are the first evidence of transcriptional regulation of the assembly chaperone genes by Rpn4 and indicate that Rpn4 activates transcription of the assembly chaperone genes even though they do not have either PACE or PACE^{var} sequences.

The reason why only these three chaperone genes, but not other chaperone genes, are highly responsive to Rpn4 is unclear. A possible explanation for this is that a large amount of Pba1, Pba2, and Ump1 proteins is required for proper assembly of the proteasome, since Pba1, Pba2, and Ump1 have been shown to be degraded during proteasome biogenesis, whereas the other six chaperones are recycled [4–6]. Alternatively, an excess of the six chaperones might be a disadvantage to cells. To test this possibility, we examined the effect of overexpression of Rpn14 or Nas6 in wild-type yeast cells. Expression of Rpn14 or Nas6 did not seem to affect the amount of the CP and the RP, as revealed by Western blot for β5/Pre2 and Rpn2 (Fig. 1E). However, overexpression of these proteins resulted in a decrease of proteasome activity in the 26S fractions (fractions 24–28) and an increase of proteasome activity in 20S fractions (fractions 14–18) (Fig. 1F, upper panel). Immunoblot analysis showed that free 20S CP, as revealed by blotting for β5/Pre2, and free 19S RP, as revealed by blotting for Rpn2, were increased when Nas6 or Rpn14 were overexpressed (Fig. 1F, lower panel). These results suggest that the association between the CP and the RP is inhibited when an excess amount of these chaperones, Nas6 and Rpn14, exists in cells. Consistent with this, Nas6 and Rpn14 have been shown to bind to the C-terminal regions of Rpt3 and Rpt6, respectively, and to have a role in preventing association of premature RP to the CP [26–28]. Therefore, induction of the assembly chaperones by Rpn4 might be optimized so that their protein levels

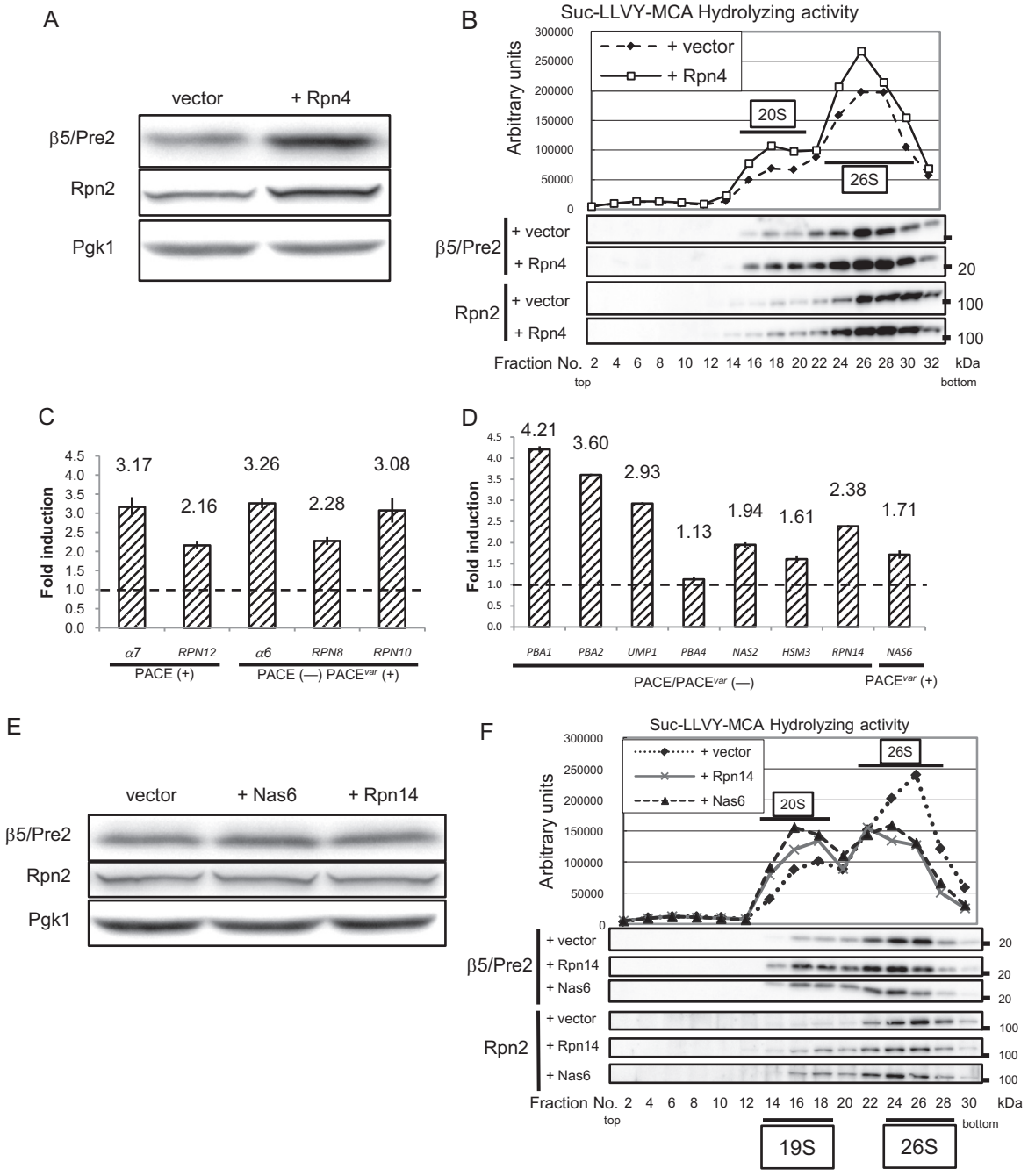

Fig. 1. Rpn4 induces properly assembled proteasomes. (A) Wild-type yeast cells with pYF5-*RPN4* or control vector were collected 2 h after Rpn4 induction by addition of galactose. Fifteen micrograms of protein were used for Western blot analysis using the indicated antibodies. Pgk1 was used for loading control. (B) Wild-type yeast cells with pYF5-*RPN4* or control vector were collected 2 h after Rpn4 induction by addition of galactose. Two milligrams of protein were separated into 32 fractions by 8–32% glycerol gradient centrifugation. Even-numbered fractions were assayed for Suc-LLVY-MCA hydrolyzing activity and subjected to SDS–PAGE, followed by Western blot analysis using the indicated antibodies. Representative data from three independent experiments are shown. (C) Wild-type cells with pYF5-*RPN4* or control vector were collected 30 min after addition of galactose to the medium. mRNAs of the indicated genes were quantified by quantitative RT-PCR (qPCR) with or without induction of Rpn4. Each mRNA was normalized to that of *ACT1*, and the fold increase of each mRNA by Rpn4 induction is shown. Mean ± SD from three independent experiments. Dashed line indicates uninduced conditions. (D) qPCR analysis was performed as in (C). (E) Western blot analysis was performed using wild-type cells with pYF5-*NAS6*, pYF5-*RPN14*, or control vector as in (A). (F) Glycerol gradient, Suc-LLVY hydrolyzing, and Western blot analyses were performed using wild-type cells with pYF5-*NAS6*, pYF5-*RPN14*, or control vector as in (B). Representative data from three independent experiments are shown.

are sufficient but not in excess for proper assembly of the 26S proteasome.

3.4. A possible minimum sequence "PACE-core" for recognition by Rpn4

The promoter regions of *PBA1*, *PBA2*, and *UMP1* do not contain sequences that exactly fit either the consensus sequences reported in previous studies or the motif described in the JASPAR database for Rpn4-binding [15,16,24,25,29]. However, a closer look at the promoter regions of the chaperone genes identified a hexamer consensus sequence 5′-(A/G)GTGGC-3′, which resembles, but differs from PACE/PACE^{var} and even does not fit the JASPAR motif 5′-(A/G/T)G(T/G)GGC(A/G)-3′ in that it lacks the seventh consensus base (A/G) (Table 3, shaded). We speculated that this hexamer motif might be a minimum requirement for recognition by Rpn4, and

Table 3
Authentic PACE, variant PACE, and PACE-core sequences in the promoter regions of proteasome subunit and assembly chaperone genes.

Gene name	PACE/PACE^{var}/PACE^{core}	Distance	Gene name	PACE/PACE^{var}/PACE^{core}	Distance
α1/SCL1	AA**TTTGCCACC**GT	100–108	*RPN1*	AC**GGTGGCAAA**AG	137–145
α2/PRE8	AA**GGTGGCAAA**AT	112–120	*RPN2*	TC**GGTGGCAAA**AC	111–119
α3/PRE9	CA**GGTGGCAAA**AA	151–159	*RPN3*	CA**TTTGCCACC**CA	87–95
α4/PRE6	CT**TTTGCCACC**GG	101–109	*RPN5*	TC**GGTGGCAAA**AT	130–138
α5/PUP2	CC**GGTGGCAAA**AG	96–104	*RPN6*	AG**GGTGGCAAA**TG	105–113
α6/PRE5	AG*AGTGGCAAA*AT	139–147	*RPN7*	AG**TTTGCCACC**CA	146–154
	AAGACGCCACTAC	87–92	*RPN8*	TC*AGTGGCAAA*TC	163–171
α7/PRE10	ATAGTGGCGGTAG	93–98	*RPN9*	CC**GGTGGCAAA**AG	101–109
	TC**GGTGGCAAA**AA	120–128	*RPN10*	TTGTTGCCACTTC	174–179
β1/PRE3	CC**GGTGGCAAA**AA	110–118		TT*TTCGCCACC*GG	93–101
β2/PUP1	CG*AGTGGCAAA*AT	150–158	*RPN11*	AA**GGTGGCAAA**TT	116–124
β3/PUP3	CC**GGTGGCAAA**AA	175–183	*RPN12*	TA**TTTGCCACC**CA	89–97
	AGAAGGCCACCTG	328–333	*RPN13*	GA*GGTGGCGAA*AG	106–114
β4/PRE10	AC**GGTGGCAAA**AA	134–142	*SEM1*	TG*GGTGGCGAA*CG	103–111
β5/PRE2	AG**GGTGGCAAA**AC	147–155		AGAGTGGCAATAA	37–42
β6/PRE7	AG**GGTGGCAAA**AA	79–87	*PBA1*	TAGGTGGCAACAG	70–75
β7/PRE4	AA**TTTGCCACC**TT	103–111	*PBA2/ADD66*	TTAGTGGCCACAT	299–304
RPT1	AT**TTTGCCACC**CG	147–155		AAAAAGCCACCTA	69–74
RPT2	CC**GGTGGCAAA**TG	123–131	*PBA3/IRC25*		
RPT3	AA**GGTGGCAAA**AT	168–176	*PBA4/POC4*		
RPT4	AA**TTTGCCACC**GA	104–112	*UMP1*	GCAGTGGCAATTT	108–113
RPT5	TTAGTGGCTGATA	317–322		TAATGGCCACTTG	216–221
	GT**GGTGGCAAA**AA	156–164	*NAS2*		
RPT6	GA**GGTGGCAAA**AG	76–84	*NAS6*	AG*TTTGCCACT*CT	307–315
				ATGGGGCCACTCA	176–181
			HSM3		
			RPN14		

Authentic PACE, bold; variant PACE, italicized and underlined; PACE-core, shaded.
Distances from initiation codons are shown.

hereafter we refer to this sequence as the "PACE-core" (PACE^{core}) sequence. Moderate induction of other chaperones despite the absence of the PACE^{core} sequences could be due to other transcriptional factors induced by Rpn4.

We then searched the yeast genome for PACE^{core} and found 584 genes that have PACE^{core} sequences within 350 bp upstream of their open reading frames (Supplementary Table 1). For further analysis, we chose several PACE^{core}-containing genes and examined whether their expression levels were increased by Rpn4. Ddi1 is a shuttle factor that delivers ubiquitinated proteins to the proteasome [30]. Get3 is a central component of the guided entry of tail-anchored proteins (GET) pathway that is required for membrane insertion of tail-anchored proteins and has recently been shown to be involved in CP assembly [31,32]. Prb1 is a vacuolar protease that functions in vacuolar protein degradation such as autophagy [33]. Bni1 is a member of the formin protein family that determines cell polarity by assembling linear actin cables [34,35]. Hsc82 and Hsp82 are molecular chaperones of the HSP90 family [36]. Along with these PACE^{core}-containing genes, *UBP6*, which has a PACE^{var} motif, was also examined as a reference. Rpn4 overexpression markedly enhanced the expression of *DDI1* and *PRB1* and moderately increased the expression of *UBP6*, *PBA2*, *GET3*, and *HSP82* (Fig. 2A). This result supports the assumption that the PACE^{core} sequences function as an Rpn4-responsive element. Strong induction of *DDI1* and *PRB1* by Rpn4 is reasonable: Under stress conditions, a large amount of shuttling factor Ddi1 is required for efficient delivery of substrate proteins to the proteasome. Induction of *PRB1* may reflect compensatory upregulation of vacuolar protein degradation, which may be necessary for the disposal of aberrant proteins and for maintenance of the amino acid pool required for cell survival under proteasome impairment [37]. In addition, we found that *GET3*, which participates in CP assembly [32], also contained PACE^{core} in the promoter and was induced by Rpn4. This observation supports the importance of PACE^{core} for proteasome assembly.

To see whether Rpn4 directly recognizes PACE^{core} sequences, chromatin immunoprecipitation (ChIP) assay was performed. Quantitative PCR analysis of ChIP-derived DNA fragments immunoprecipitated with anti-Flag antibody showed that the promoter regions of PACE^{core} (+) *PBA2* as well as PACE (+) *RPN12* were moderately but significantly enriched compared with *HSM3*, which contains neither PACE nor PACE^{core} sequence (Fig. 2B). This result supports the view that PACE^{core} sequences function as an Rpn4-binding element.

Although PACE^{core} sequences functions as Rpn4-binding sequences, some of the genes with PACE^{core} do not seem to be strongly induced by Rpn4 overexpression, such as *BNI1* and *HSC82* (Fig. 2A). This may be due to binding of an as yet unidentified transcription repressor to the promoter regions and raises the possibility that a multilayered mechanism might exist in Rpn4-dependent transcription regulation.

3.5. The PACE-core does not substitute the PACE in the proteasome subunit promoter under stress conditions

We next tested whether there was a difference in the ability to induce Rpn4-dependent gene expression between the PACE and the PACE^{core} sequences. To this end, we constructed low-copy plasmids that express *α4* and *RPN6* from their native promoters (designated pRS315-*α4* and pRS315-*RPN6*, respectively). We also made plasmids in which the authentic PACE sequence (5′-GGTGGC*AAA*-3′) in the promoter regions of *α4* or *RPN6* in pRS315-*α4* and pRS315-*RPN6* were replaced with the PACE^{core} sequence of the *PBA2* promoter (5′-GGTGGC*TTT*-3′). These plasmids were designated pRS315-*α4** and pRS315-*RPN6**, respectively. In order to exclude transcripts of *α4* and *RPN6* from endogenous alleles, we introduced pRS315-*α4* and pRS315-*α4** into *α4Δ* (designated *α4*- and *α4**-cells) and pRS315-*RPN6* and pRS315-*RPN6** into *rpn6Δ* strains (designated *RPN6*- and *RPN6**-cells, respectively) and observed whether replacement of the PACE with the PACE^{core} affected the

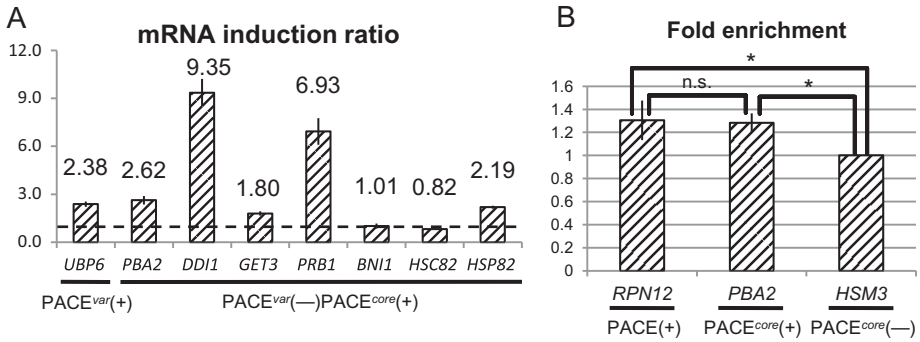

Fig. 2. Rpn4 directly induces PACE-core containing genes. (A) Wild-type cells with pYF5-*RPN4* or control vector were collected 30 min after addition of galactose to the medium. mRNAs of the indicated genes were quantified by quantitative RT-PCR with or without induction of Rpn4. Each mRNA was normalized to that of *ACT1*, and the fold increase of each mRNA by Rpn4 induction is shown. Mean ± SD from three independent experiments. Dashed line indicates uninduced conditions. (B) Wild-type cells with pUZ251-*RPN4* (3xFlag-Rpn4) or control vector were fixed and harvested 30 min after addition of galactose to the medium, and ChIP assays were performed against Flag epitope. Co-immunoprecipitated DNA was quantified by quantitative PCR and normalized to the amount of input DNA. Fold-enrichment by Rpn4 was calculated and compared to that of *HSM3*. Data were statistically analyzed by the Mann–Whitney *U* test. $^{*}p < 0.05$. n.s.: not significant.

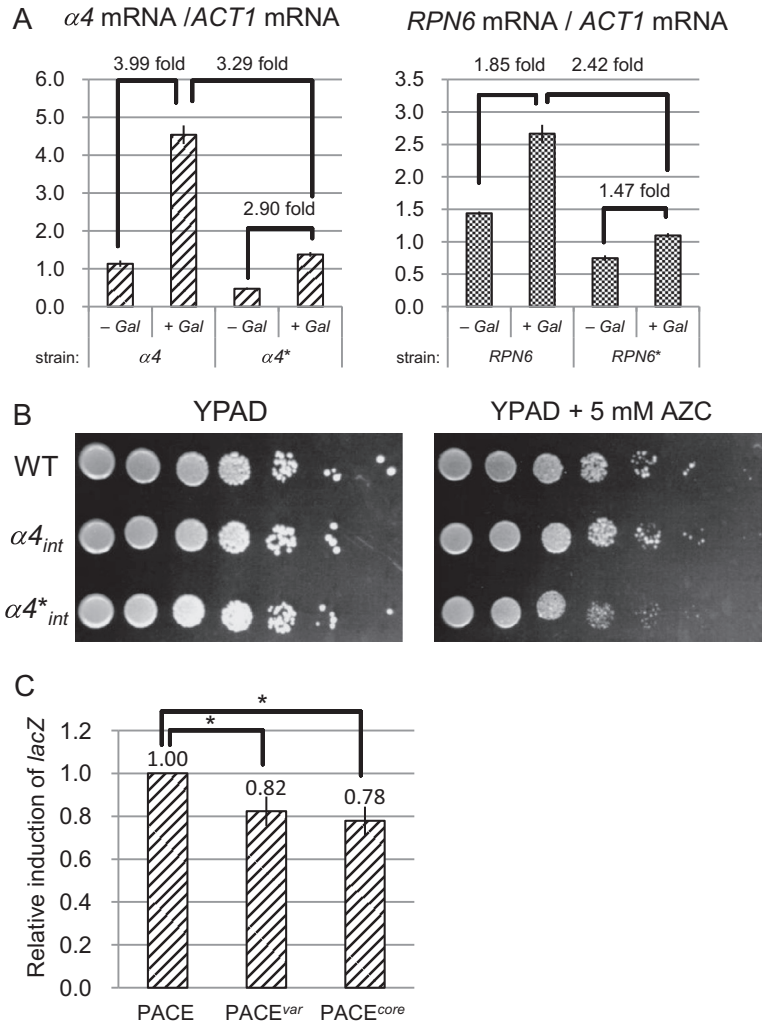

Fig. 3. PACE-core does not substitute authentic PACE of proteasome subunits. (A) Haploid *α4Δ* and *rpn6Δ* cells with wild-type (pRS315-*α4* or pRS315-*RPN6*) or PACE^{core} (pRS315-*α4** or pRS315-*RPN6**) plasmids were transformed with pYF5-*RPN4*. Cells were collected before and 30 min after addition of galactose to the media to induce Rpn4 and subjected to RNA extraction. The amount of each mRNA was normalized to that of *ACT1*. Fold increase by Rpn4 induction is shown. Mean ± SD from three independent experiments. (B) Serial fivefold dilution of haploid wild-type, *α4*$_{int}$- and *α4*$^{*}_{int}$-cells were spotted on YPAD (left) and YPAD containing 5 mM azetidin-2-carboxylic acid (AZC, right) prior to 3 days incubation at 26 °C. (C) PACE, PACE^{var}, and PACE^{core} were fused to the *CYC1* minimal promoter placed upstream of the *lacZ* gene. These constructs were introduced into Rpn4-overproducing cells, and LacZ activities were measured and normalized to that of the PACE construct. Mean ± SD from three independent experiments is shown.

expression levels of *α4* and *RPN6* mRNAs both in basal and Rpn4-induced conditions.

The amount of *α4* and *RPN6* mRNAs in *α4**- and *RPN6**-cells were smaller than those in *α4*- and *RPN6*-cells under basal culture conditions (Fig. 3A, −Gal). When Rpn4 expression was induced, *α4* and *RPN6* mRNAs were induced in all *α4*-, *α4**-, *RPN6*-, and *RPN6**-cells. However, induction was weaker in cells containing the PACE^{core} constructs than those in cells containing the PACE constructs. Consequently, the amount of mRNA for *α4* and *RPN6* was approximately 2 to 3 times higher in *α4*- and *RPN6*-cells than in *α4**- and *RPN6**-cells (Fig. 3A, +Gal). Therefore, we concluded that the PACE sequence is more potent than the PACE^{core} sequence with respect to Rpn4-mediated gene expression, even though both the PACE and PACE^{core} respond to Rpn4.

To test whether the weak responsiveness of PACE^{core} sequence to Rpn4 might render cells more sensitive to proteotoxic stress, we made *α4**$_{int}$-cells in which the genomic PACE sequence of the *α4* promoter was replaced with the PACE^{core} sequence of *PBA2*. As a control, we also made *α4*$_{int}$-cells in which the genomic PACE sequence of the *α4* promoter was replaced with the PACE sequence of *α4*. Azetidin-2-carboxylic acid (AZC) is a proline analog, the incorporation of which increases misfolded proteins, and thus yeast strains that are defective in proteasomal degradation show higher sensitivity to AZC than wild-type strains [38,39]. *α4**$_{int}$-cells grew as well as *α4*$_{int}$-cells and wild-type cells under normal conditions. However, in the presence of AZC, the colonies of the *α4**$_{int}$-cells were smaller than those of the wild-type and *α4*$_{int}$-cells (Fig. 3B). These results suggest that the authentic PACE or PACE^{var} sequences in the promoter regions of proteasome genes are required to maintain sufficient proteasome activity to degrade abnormal proteins under proteotoxic conditions and that the PACE^{core} sequence does not substitute for the authentic PACE sequence in a proteasome subunit gene.

To further confirm that the differences in Rpn4-recognition sequences result in different expression levels of the downstream gene, we employed single reporter constructs, in which either the PACE, PACE^{var}, or PACE^{core} sequence was introduced 5′-adjacent to the *CYC1* minimal promoter placed upstream of the *lacZ* gene [40–42]. These constructs were introduced into Rpn4-overproducing cells, and LacZ activities were measured. The induction of *lacZ* was significantly smaller in the cells expressing the PACE^{var} and PACE^{core} constructs than in those expressing the authentic PACE construct (Fig. 3C). These results are consistent with the results in Fig. 3B and further suggest that differences in Rpn4-binding sequences affect the expression levels of the downstream genes, where the authentic PACE is the most potent.

In summary, Rpn4 mediates transcription of a wider range of genes than has been previously recognized. The finding of the PACE^{core} expands the role of Rpn4 when proteasome activity is compromised and thus may contribute to our understanding of how cells manage to maintain proteostasis under conditions of cellular stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.025.

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